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# Expression of bystin in reactive astrocytes induced by ischemia/reperfusion and chemical hypoxia in vitro

Du Fang [a,b], Zhu Li [a,c,*], Qian Zhong-ming [a,c,*], Wu Xiao Mei [a,b], Yung Wing Ho [b], Xu Wei Yuan [c], Ke Ya [b,*]

[a] Department of Neurobiology and Neurochemistry, Institute for Nautical Medicine and Jiangsu Key Laboratory of Neurogeneration, Nantong University, Nantong 226001, PR China
[b] Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
[c] Laboratory of Brain Iron Metabolism, Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University and National Key Laboratory of Chinese Medicine and Molecular Pharmacology (Shenzhen), Hong Kong

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ABSTRACT

In this study, we investigated the effects of ischemia/reperfusion and chemical hypoxia on the morphology, cell viability and expression of bystin and glial fibrillary acidic protein (GFAP) in primary cultured astrocytes which were prepared by the subculture method. The astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were first exposed to 1% O2 and then to 21% O2 (normoxia), or treated with different concentrations of $CoCl_2$ or $NaN_3$ for different periods. Relevant observations and measurements were then conducted. The findings showed that treatment with 1% $O_2$ for 0.5 or 3 h could induce a characteristic 'reactive' morphology and a significant increase in cell viability and total protein amount. The western blot analysis showed that treatment with 1% $O_2$ for 0.5 or 3 h also induced a significant increase in the expression of bystin and that the response of bystin to mild ischemia was much more sensitive than that of GFAP. Similar results were also found in the cells treated with mild chemical hypoxia. The data demonstrated for the first time that mild ischemia and hypoxia could activate astrocytes and that bystin is a much more sensitive marker in activated astrocytes induced by ischemia and hypoxia as compared to GFAP. The significant up-regulation of bystin suggests that bystin may play an important role in the activation of astrocytes as well as in the neuroprotective role of hypoxic and ischemic preconditioning.
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## 1. Introduction

It is well known that astrocytes have the critical functions of the maintenance of ionic homeostasis, prevention of excitotoxicity, scavenging free radicals, provision of nutrients and growth factors, promotion of neovascularization, and support of synaptogenesis and neurogenesis that may potentially influence the outcome of ischemic injury [1]. Astrocytes become reactive (Astrogliosis) in response to many pathologies of the central nervous system (CNS), such as stroke, trauma, growth of a tumor, chemical insult or neurodegenerative disease [2,3]. Astrogliosis is characterized by astrocyte proliferation, extensive hypertrophy of nuclei, cell body, and cytoplasmic processes and an increase in immunodetectable glial fibrillary acidic protein (GFAP) [4,5].

As a member of the cytoskeletal protein family, it is believed that GFAP is important in modulating astrocytic motility and shape by providing structural stability to astrocytic processes [5]. In addition to the increased expression of GFAP with the activation of astrocytes, it has been recently reported that bystin, a protein potentially involved in embryo implantation [6,7], is markedly up-regulated in the reactive astrocytes of both 6-hydrodopamine-lesioned nigrostriatum and stab-lesioned cerebral cortex of adult rats in vivo and in postnatal cortical astrocytes treated with pro-inflammatory mediators lipopolysaccharide and interleukin-1b in vitro [8]. It has been also suggested [8] that bystin acts as a novel marker for reactive astrocytes in the adult rat brain following injury and its expression may be involved in the differentiation of reactive astrocytes during the inflammatory processes.

However, it is unknown whether the expression of bystin is also increased in the reactive astrocytes induced by other pathologies such as ischemia and hypoxia because it has been suggested that different types of injury may induce different astrocyte responses [9]. It is also unknown whether the increased expression of bystin is a common characteristic of astrogliosis. In this study, we therefore investigated the effects of ischemia and chemical hypoxia on the morphology, cell ability and expression of bystin as well as GFAP in primary cultured astrocytes. Our findings demonstrated that compared with GFAP, bystin is a more sensitive marker for the reactive astrocytes induced by ischemia/reperfusion and chemical hypoxia in vitro, and suggested that the increased expression of bystin might be one of the common characteristics of astrogliosis.

* Corresponding authors. Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong. Tel.: +852 2609 6780.
*E-mail addresses:* zhulili65@yahoo.com (Z. Li), bczmqian@polyu.edu.hk (Q. Zhong-ming), yake@cuhk.edu.hk (K. Ya).

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## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. The astrocyte marker glial fibrillary acidic protein (GFAP) was purchased from Chemicon International Ltd, UK and the mouse anti-bystin antibody was a gift from Jiawei Zhou, Shanghai Institutes for Biological Sciences, China. Fetal bovine serum was obtained from Hyclone, Logan, UT, USA and Dulbecco's modified Eagle's medium from Gibco-BRL, Carlsbad, California, USA. The DC Protein Assay kit was bought from Bio-Rad Laboratories, Hercules, CA, USA and horseradish (HRP)-conjugated secondary antibodies from Pierce Chemical Company, USA.

### 2.2. Primary culture of mouse cerebrocortical astrocytes

All experiments were carried out with mouse cerebrocortical astrocytes. The cells were prepared from newborn ICR mice by a procedure described previously [10,11]. Briefly, cerebral cortices were cut into small cubes (<1 $mm^3$) and digested with 0.25% trypsin (Sigma, USA) for 30 min at 37°C. Trypsinization was terminated by an addition of Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), followed by mechanical trituration with a flame-polished Pasteur glass pipette. Cell suspensions were sieved through a 40 μm cell strainer and the filtrate was allowed pre-adherence for 30 min to remove any contamination from fibroblast before being seeded at a density of approximately one cerebrum per 50 $cm^2$ flask (Corning, USA) or six-well plates (NUNC, Denmark). The plated cells were incubated in a 5% $CO_2$ incubator at 37 °C. After the cultures reached confluence (12–14 days), they were subcultured 3 times every 4 days, and were allowed pre-adherence for 30 min before being seeded in each subculture. The purity of the astrocytes cultures was assessed by staining for the astrocyte marker glial fibrillary acidic protein (GFAP) (Chemicon International Ltd, UK), which was approximately 99%. They were cultured for another 7 days after the last subculture before being used.

### 2.3. Experimental design

To determine the effects of ischemia/reperfusion on cell viabilities and the expression of bystin and GFAP, the astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were exposed to 1% O2 in a dedicated incubator (NAPCO 7101FC-1) with 94% N2 and 5% CO2 at 37 °C for 0, 0.5, 3, 6, 9, 12, 18 or 24 h, and then exposed to 21% O2 (normoxia) for 24 h [12]. To investigate the effects of chemical hypoxia on cell viabilities and the expression of bystin and GFAP, the cells were treated with $CoCl_2$ (0, 0.5, 1, 2.5 or 5 mM) for 24 h, or $NaN_3$ (0, 1 or 10 mM) for 15, 30 or 60 min respectively. Then the relevant measurements were conducted.

### 2.4. Assessment of cell viability

The cell viabilities of astrocytes that received different treatments were measured using an MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide) assay as described by He et al [13]. Briefly, a total of 25 μL MTT (1 g/L in PBS) was added to each well before the conduction of incubation at 37 °C for 4 h. The assay was stopped by the addition of a 100 μL lysis buffer (20% SDS in 50% *N'N*-dimethylformamide, pH 4.7). Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-tek, USA) and the results were expressed as a percentage of absorbance measured in the control cells.

### 2.5. Western blot analysis

Astrocytes receiving different treatments were washed with ice-cold PBS and the proteins were extracted with 150 μl lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml of heat activated sodium ortovanadate. After centrifugation at 10,000 ×*g* for 15 min at 4 °C, the supernatant was collected. Protein content was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA). A total of 30 mcg proteins was boiled in protein loading buffer for 5 min, separated on a 10% SDS-polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. Nonspecific binding was inhibited by incubation in TBST [20 mM Tris-buffered saline (pH 7.5) with 0.1% Tween 20] containing 5% nonfat dried milk for 2 h at room temperature. The membranes were incubated with monoclonal antibodies against GFAP and bystin (1:10000 in TBST containing 5% milk) overnight at 4°C. After three washes with TBST, the membranes were incubated for 2 h with horseradish (HRP)-conjugated secondary antibodies (Pierce Chemical Company, USA) and developed using enhanced chemiluminescence (ECL western blotting analysis system kit, Amersham Biosciences, England). The blot was detected using Kodak XAR-5 film for autoradiography. To ensure even loading of the samples, the same membrane was probed with anti-mouse β-actin monoclonal antibody (Sigma-Aldrich, MO) at a 1:10000 dilution.

### 2.6. Statistical analysis

The statistical analyses were performed using SPSS 10.0. Data are presented as mean±SEM. The difference between means was determined by One-Way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. A probability value of $p<0.05$ was taken to be statistically significant.

## 3. Results

### 3.1. Effects of ischemia/reperfusion on the viability and morphology of astrocytes

The viability of the cultured astrocytes treated with ischemia/reperfusion was determined using an MTT assay. The MTT assay results, as shown in Fig. 1, indicated that the treatment of the cells

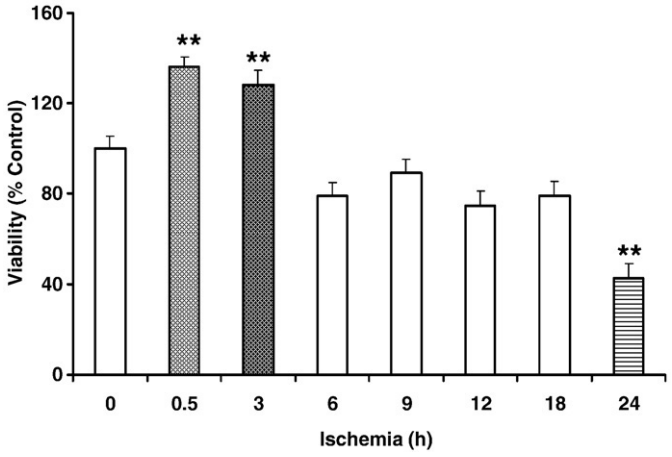


**Fig. 1.** Effects of ischemia/reperfusion on the viability of astrocytes. The astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were exposed to 1% O2 for 0, 0.5, 3, 6, 9, 12, 18 or 24 h and subsequently to 21% O2 (normoxia) for 24 h. The cell viabilities were then measured using an MTT assay as described in 'Materials and Methods'. Data are means±SEM ($n=18$). **$p<0.01$ vs. the control.

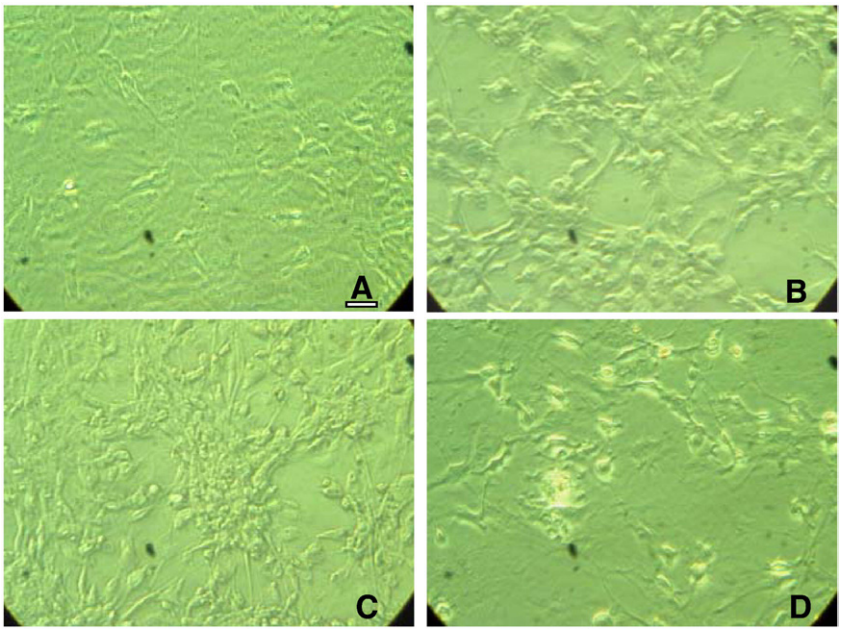


**Fig. 2.** Effects of ischemia/reperfusion on the morphology of astrocytes. The astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were exposed to 1% O2 for 0, 0.5, 3 or 24 h and subsequently to 21% O2 (normoxia) for 24 h. The morphological changes were then observed under a light microscope. (A) the Control; (B) 0.5 h ischemia; (C) 3 h ischemia; and (D) 24 h ischemia (scale bar=40 mM).

with 0.5 or 3 h ischemia and 24 h reperfusion led to a significant increase in cell viabilities ($p<0.01$, vs. the control). No significant difference in cell viabilities was found between the controls and the cells treated with 6, 9, 12 or 18 h ischemia and 24 h reperfusion. However, cell viabilities were significantly decreased in the cells treated with 24 h ischemia and 24 h reperfusion ($p<0.01$, vs. the control).

In agreement with the results of cell viability, the treatment of the astrocytes with 0.5 (Fig. 2B) or 3 h (Fig. 2C) ischemia and 24 h reperfusion induced a characteristic 'reactive' morphology, ie. hypertrophic processes and stellate-shaped cell bodies. Treatment with 24 h (Fig. 2D) ischemia and 24 h reperfusion also induced a significant change in cell morphology. Cell bodies began to shrink and processes decurtated or even disappeared. Relics of the dead cells could be found under a light microscope (Fig. 2D).

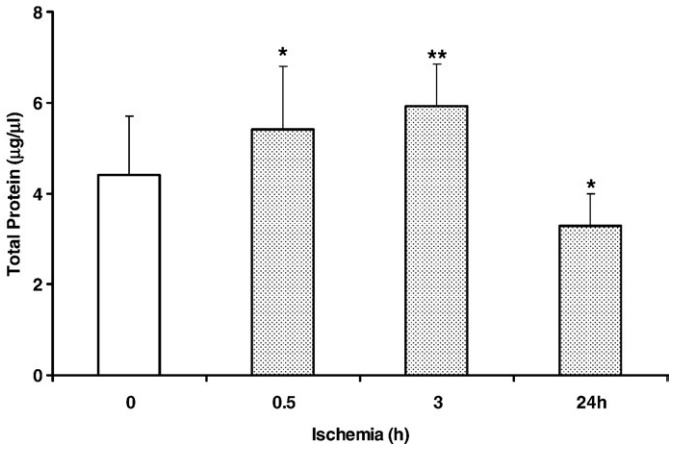


**Fig. 3.** Total protein amount of astrocytes treated with ischemia/reperfusion. The astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were exposed to 1% O2 for 0, 0.5, 3 or 24 h and subsequently to 21% O2 (normoxia) for 24 h. Total protein amount of astrocytes was then determined using the DC Protein Assay kit as described in 'Materials and Methods'. Data are means±SEM ($n=6$). *$p<0.05$, **$p<0.01$ vs. the control.

### *3.2. Total protein amount of astrocytes treated with ischemia/reperfusion*

We then measured the total protein amount of astrocytes treated with 0.5, 3 or 24 h ischemia and 24 h reperfusion. The results (Fig. 3) showed that the total protein amount in the cells treated with 0.5 or

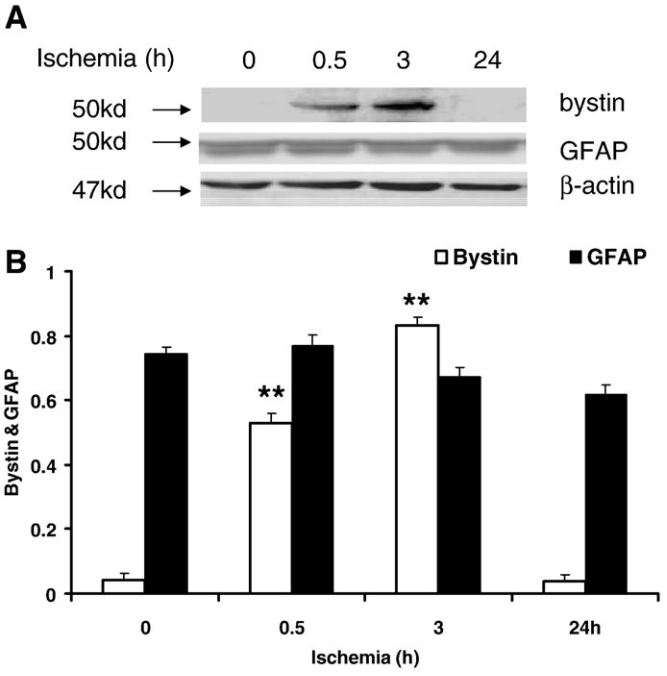


**Fig. 4.** Effects of ischemia/reperfusion on expression of GFAP and bystin. The astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation, ischemic cells) were exposed to 1% O2 for 0, 3 or 24 h and subsequently to 21% O2 (normoxia) for 24 h. Western blot analysis were then conducted as described in 'Materials and Methods'. (A) A representative experiment of Western blot of bystin, GFAP and β-actin. (B) Quantification of expression of bystin, GFAP proteins in astrocytes. Expression values were normalized for β-actin and the data were presented as Mean±SEM ($n=5$). **$p<0.01$ versus the control (bystin).

3 ischemia and 24 h reperfusion was significantly higher ($p<0.05$ or 0.01, vs. the control) and in the cells treated with 24 h ischemia and 24 h reperfusion was significantly lower ($p<0.05$, vs. the control) than that in the control.

### 3.3. Effects of ischemia/reperfusion on expression of GFAP and bystin

To determine the effects of ischemia/reperfusion on the expression of bystin and GFAP, the astrocytes in Hank's medium without glucose and serum were exposed to 1% O2 at 37 °C for 0, 0.5, 3 or 24 h, and then to 21% O2 for 24 h. Western blot results showed that ischemia/reperfusion did not induce any significant changes in the expression of GFAP (Fig. 4). There were no significant differences in the levels of GFAP in the cells treated with ischemia for 0, 0.5, 3 or 24 h. However, treatment of the cells with ischemia for 0.5 and for 3 h induced a significant increase in the expression of bystin ($p<0.01$, vs. the control). No difference was found in the expression of bystin between the cells treated with or without 24 h ischemia and 24 h reperfusion.

### 3.4. Effects of chemical hypoxia on cell viability and the expression of GFAP and bystin in astrocytes

We subsequently investigated the effects of chemical hypoxia on cell viability and expression of GFAP and bystin in astrocytes by treating the cells with 1 mM $NaN_3$ for 0, 15, 30 or 60 min, and 10 mM for 60 min, and $CoCl_2$ (0, 0.5, 1, 2.5 or 5 mM) for 24 h, respectively. The MTT assay results indicated that treatment of the cells with lower concentrations of $NaN_3$ (1 mM) (Fig. 5A) or $CoCl_2$ (0, 0.5, 1 or 2.5 mM) (Fig. 6A) induced a significant increase in cell viabilities ($p<0.05$ or 0.01, vs. the control). However, cell viabilities were significantly decreased in the cells treated with higher concentrations of $NaN_3$ (10 mM) (Fig. 5A) or $CoCl_2$ (5 mM) (Fig. 6A) ($p<0.01$ or 0.001, vs. the control).

Same as those found in the cells treated with ischemia/reperfusion, Western blot results showed that treatments of the cells with different concentrations of $NaN_3$ or $CoCl_2$ did not induce any significant effects on the expression of GFAP (Figs. 5B, C, 6B, C). There were no significant differences in the levels of GFAP in the cells that received different treatments. However, the expression of bystin was significantly

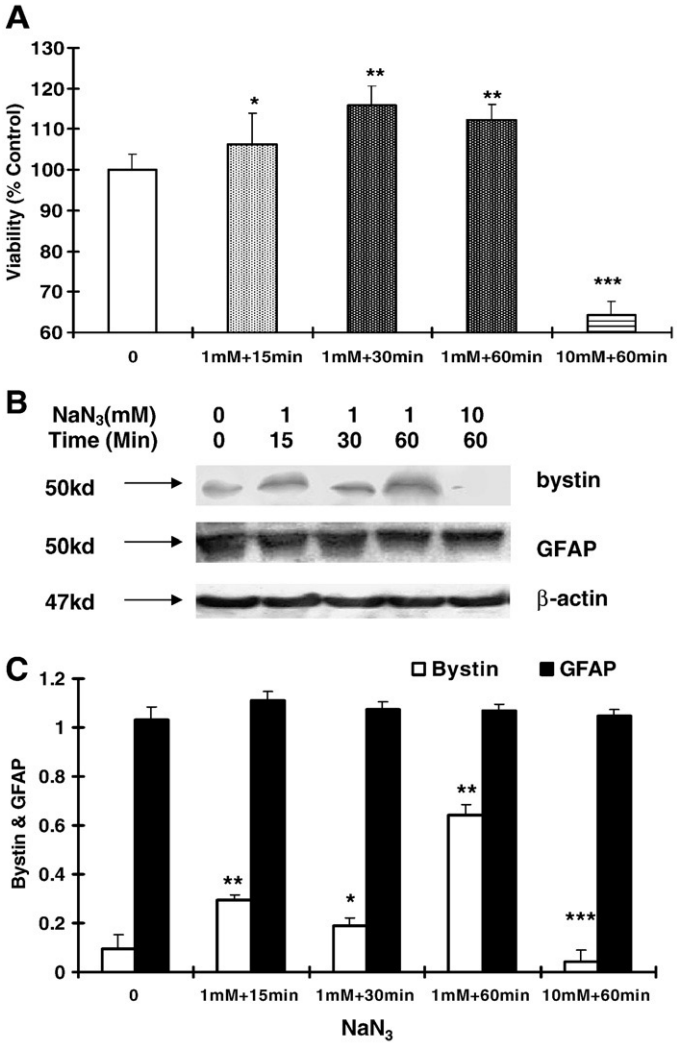


**Fig. 5.** Effects of $NaN_3$ on cell viability and expression of GFAP and bystin in astrocytes. The astrocytes were treated with 1 mM $NaN_3$ for 0, 15, 30 or 60 min, or 10 mM for 60 min. The cell viabilities and the expression of proteins were then measured using an MTT assay and Western blot analysis as described in 'Materials and Methods'. (A) Cell viability (the data were presented as Mean±SEM ($n=5$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus the control); (B) A representative experiment of Western blot of bystin, GFAP and β-actin; and (C) Quantification of expression of bystin and GFAP proteins in astrocytes. Expression values were normalized for β-actin and the data were presented as Mean±SEM ($n=5$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus the control (bystin).

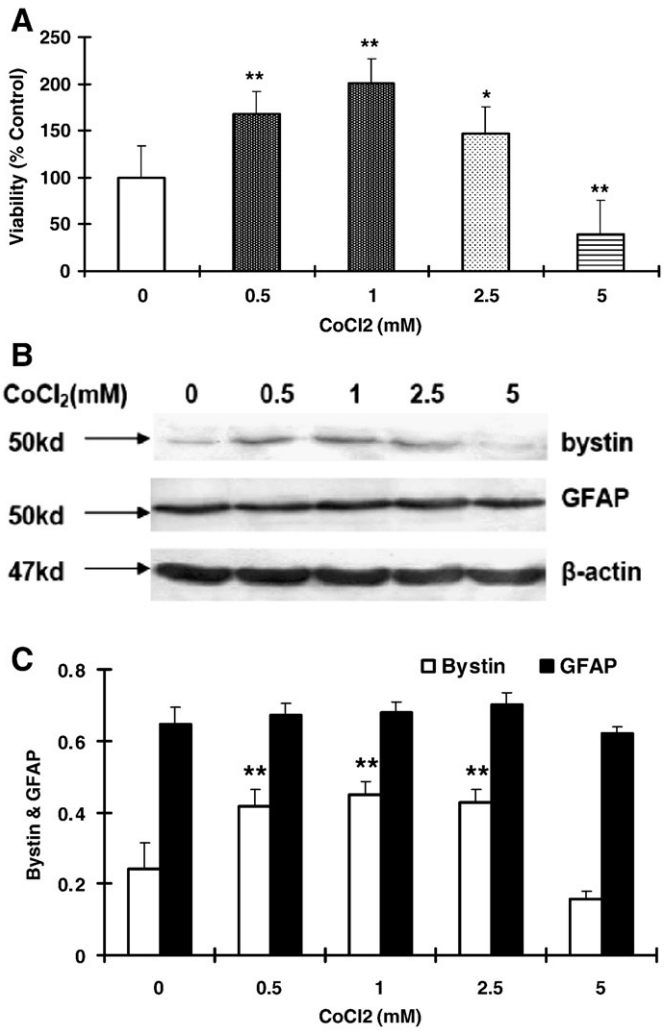


**Fig. 6.** Effects of $CoCl_2$ on cell viability and expression of GFAP and bystin in astrocytes. The astrocytes were treated with $CoCl_2$ (0, 0.5, 1, 2.5 or 5 mM) for 24 h. The cell viabilities and the expression of proteins were then measured using an MTT assay and Western blot analysis as described in 'Materials and Methods'. (A) Cell viability (the data were presented as Mean±SEM ($n$=5). *$p$<0.05, **$p$<0.01 versus the control); (B) A representative experiment of Western blot of bystin, GFAP and β-actin; and (C) Quantification of expression of bystin and GFAP proteins in astrocytes. Expression values were normalized for β-actin and the data were presented as Mean±SEM ($n$=5). **$p$<0.01 versus the control (bystin).

increased in the cells treated with lower concentrations of $NaN_3$ (1 mM) (Fig. 5B, C) or $CoCl_2$ (0, 0.5, 1 or 2.5 mM) (Fig. 6B, C) and decreased in the cells treated with higher concentrations of $NaN_3$ (10 mM) or $CoCl_2$ (5 mM).

## 4. Discussion

In the present study, we demonstrated that treatment of the mouse cerebrocortical astrocytes with mild ischemia (1% $O_2$ for 0.5 or 3 h) could induce a significant increase in cell viability, total protein amount and a characteristic 'reactive' morphology (hypertrophic processes and stellate-shaped cell bodies). Similar results were also found in the cells treated with mild chemical hypoxia (1 mM of $NaN_3$ for 0, 15, 30, 60 min or 0.5, 1, 2.5 mM of $CoCl_2$ for 24 h). These findings clearly showed that mild ischemia and hypoxia could activate astrocytes under our experimental conditions. Our study also demonstrated that the activated astrocytes or reactive astrocytes have a significantly increased expression of bystin (Fig. 7). Compared with the effects of ischemia and hypoxia on the expression of GFAP, bystin is a much more sensitive marker for reactive astrocytes induced by ischemia and chemical hypoxia in vitro. The data suggested that bystin might be a useful marker for stroke and a key factor in the formation of reactive astrocytes in the brain under ischemia and hypoxia.

To our knowledge, this report is the first study on the effects of ischemia/reperfusion and chemical hypoxia on the expression of bystin and GFAP in astrocytes. Our findings demonstrated that the response of GFAP is much less sensitive than that of bystin to ischemia and chemical hypoxia in vitro. Western blot results demonstrated that ischemia did not induce any significant changes in the expression of GFAP. There were no significant differences in the levels of GFAP in the cells treated with ischemia for 0, 0.5, 3 or 24 h. GFAP is a major type of intermediate filament (IF). A significant increase in GFAP is almost universally recognized as a marker of astrogliosis [14]. Norton et al [9] have suggested that different types of injury induce different astrocyte responses, characterized in terms of the time course, the involvement of the contralateral side, and whether GFAP mRNA and/or protein are increased. The different types of injury used in the present study and others [4,5,8,15,16] might be one of the major reasons for the differences in results on the changes in the expression of GFAP.

In contrast to GFAP, our data demonstrated that treatment of the cells with ischemia for 0.5 and for 3 h induced a significant increase in the expression of bystin. Similar results were also found in the cells treated with mild chemical hypoxia. Human bystin is a 306-amino acid cytoplasmic protein which mediates a unique homophilic cell adhesion between trophoblast and endometrial epithelial cells at their respective apical cell surfaces [6,7,17]. Recent studies suggested that this protein may play multiple roles in mammalian cells. A function is to facilitate ribosome biogenesis required for cell growth [18]. It has also been suggested that bystin expressed by reactive astrocytes may be involved in their differentiation during the inflammatory processes following brain injury induced by both 6-hydrodopamine-lesioned rat nigrostriatum and stab-lesioned cerebral cortex in adult rats [8]. In addition, a potential connection between bystin expression and adult neurogenesis has also been reported [17,19]. The significant and sensitive response of bystin expression to ischemia and chemical hypoxia found in the present study strongly suggests that bystin may play an important role in the changes in astrocytes which lead to astrogliosis and also in the neuroprotective role of hypoxic and

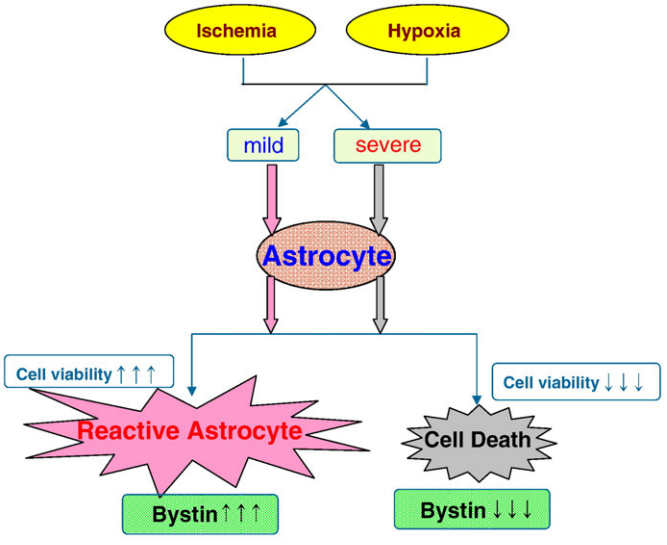


**Fig. 7.** A hypothetical scheme for the effects of ischemia and hypoxia on astrocytes. Reactive astrocytes can be induced by mild ischemia and hypoxia with a remarkable up-regulation of cell viabilities and increased expression of bystin. Severe ischemia and hypoxia will result in astrocyte death with a down-regulation of cell viabilities and bystin expression.

ischemic preconditioning. To confirm these hypotheses and to investigate the associated mechanisms, further studies are needed.

The mouse cerebrocortical astrocytes were prepared using the subculture method in the present study. Meanwhile, we noted that in a number of studies astrocytes were prepared by a method that involved a 'shaken' procedure (shaken over night once or twice) in order to remove microglia and oligodentrocytes [20–26]. We therefore compared the cell viabilities and immunocytochemical staining of GFAP in 'shaken' astrocytes with those in subcultured astrocytes. The findings showed that these two parameters in the former were significantly higher than in the latter (data not shown). This implies that shaking itself might activate astrocytes (reactive astrocytes), whereas astrocytes prepared by the subculture method are still in an "inactive state" (quiescent astrocytes). We then measured the effects of ischemia on cell viabilities in the 'shaken' astrocytes and found that there was no significant difference in cell viabilities between the 'shaken' cells treated with or without mild ischemia (1% $O_2$ for 0.5 or 3 h) (data not shown). It is completely different from that we found in subcultured cells where cell viability was significantly increased by mild ischemia. These data imply that mild ischemia could induce an increase in cell viabilities only in 'quiescent astrocytes' but not in 'reactive astrocytes'. The mild ischemia cannot induce a further increase in cell viabilities in 'reactive astrocytes'. In addition to the 'shaken' procedure, it has been reported that a serum-free condition could be an incentive to astrocytes [27], inducing activation of astrocytes. Therefore, it is possible that mild ischemia might not induce an increase in cell viabilities in the astrocytes pretreated with serum-free media.

In summary, our data demonstrated for the first time that mild ischemia and chemical hypoxia could activate mouse cerebrocortical astrocytes and that bystin is a much more sensitive marker in activated astrocytes induced by ischemia and chemical hypoxia as compared with GFAP. The significant and sensitive response of bystin expression to ischemia and chemical hypoxia suggests that bystin may play an important role in the activation of astrocytes as well as in the neuroprotective role of hypoxic and ischemic preconditioning.

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